4μ8C Inhibits Insulin Secretion Independent of IRE1α RNase Activity

Hitomi Sato¹, Yoko Shiba^{1,2*}, Yuichi Tsuchiya¹, Michiko Saito¹, and Kenji Kohno^{1**}

¹Laboratory of Molecular and Cell Genetics, Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5, Takayama, Ikoma, Nara 630-0192, Japan, ²Faculty of Science and Engineering, Iwate University, 4-3-5, Ueda, Morioka 020-8551, Iwate, Japan

ABSTRACT. IRE1α plays an important role in the unfolded protein response (UPR), which is activated by the accumulation of unfolded proteins in the endoplasmic reticulum. 4μ8C, a well-known inhibitor of IRE1α RNase activity, is commonly used to analyze IRE1α function during ER stress in cultured mammalian cells. However, the off-target effects of 4μ8C remain elusive. Pancreatic β-cells synthesize a large amount of insulin in response to high glucose stimulation, and IRE1α plays an important role in insulin secretion from pancreatic β-cells. Here, to analyze the role of IRE1α in pancreatic β-cells, we examined insulin secretion after 4μ8C treatment. Although 4μ8C inhibited insulin secretion within 2 hr, neither insulin synthesis nor maturation was inhibited by 4μ8C under the same conditions. This result prompted us to examine the precise effects of 4μ8C on insulin secretion in pancreatic β-cells. Unexpectedly, with just 5 min of treatment, 4μ8C blocked insulin secretion in cultured pancreatic β-cells as well as in pancreatic islets. Furthermore, insulin secretion was prevented by 4μ8C, even in pancreatic β-cells lacking the IRE1α RNase domain, suggesting that 4μ8C blocked the late stage of the insulin secretory process, independent of the IRE1α-XBP1 pathway. Our results indicate that 4μ8C has an off-target effect on insulin secretion in pancreatic β-cells. These findings inform the researchers in the field that the use of 4μ8C requires the special consideration for the future studies.

Key words: 4μ8C, XBP1, insulin, IRE1α, pancreatic β-cells

Introduction

Pancreatic β-cells are specialized cells that secrete insulin to regulate blood glucose levels. Preproinsulin, a precursor of insulin, is synthesized in the cytosol and co-translationally inserted into the endoplasmic reticulum (ER) (Liu *et al.*, 2014; Rhodes *et al.*, 2005; Sun *et al.*, 2015). Its signal peptide is cleaved and proinsulin is released into the lumen of the ER. Proinsulin forms three disulfide bonds in the ER, and properly folded proinsulin is then transported to the Golgi apparatus. Proinsulin is packaged into the secretory

granules and converted into mature insulin. The secretory granules fuse with the plasma membrane upon high glucose stimulation (exocytosis) and insulin is released from β-cells. High glucose stimulates insulin synthesis as well as exocytosis. Insulin biosynthesis is triggered by 2–4 mM glucose, whereas exocytosis is stimulated by more than 5 mM glucose (Sun *et al.*, 2015).

Insulin biosynthesis accounts for 10–50% of total protein synthesis in β-cells (Schuit *et al.*, 1988; Van Lommel *et al.*, 2006). High demand for insulin biosynthesis causes ER stress in β-cells, characterized by the accumulation of unfolded proteins in the ER. During ER stress, the unfolded protein response (UPR) mediates a reduction in the total protein load in the ER and increases the expression of ER chaperones and ER-associated protein degradation (ERAD) machinery to restore ER homeostasis (Korennykh and Walter, 2012; Ron and Walter, 2007; Kimata and Kohno, 2011). IRE1α is a type-I transmembrane protein localized in the ER; it plays an important role as an ER stress sensor for the UPR in mammalian cells (Tirasophon *et al.*, 1998). IRE1α has a sensor domain in the luminal region and a kinase and RNase domain in the cytoplasmic region (Korennykh and Walter, 2012). The luminal region detects

*To whom correspondence should be addressed: Yoko Shiba, Laboratory of Intracellular Traffic, Faculty of Science and Engineering, Iwate University, Bldg 6 Room 402, 4-3-5, Ueda, Morioka 020-8551, Japan.

Tel: +81-19-621-6311

E-mail: shibay@iwate-u.ac.jp

**To whom correspondence should be addressed: Kenji Kohno, Graduate School of Biological Sciences, and Institute for Research Initiatives, Nara Institute of Science and Technology, 8916-5, Takayama, Ikoma, Nara 630-0192, Japan.

Tel: +81-743-72-6207, Fax: +81-743-72-5615

E-mail: kkouno@bs.naist.jp

Abbreviations: ER, Endoplasmic Reticulum; UPR, Unfolded Protein Response; IRE1α, Inositol-Requiring Enzyme 1α; ANOVA, analysis of variance.

the accumulation of unfolded proteins and facilitates the oligomerization of IRE1 α , leading to self-phosphorylation in its kinase domain to activate RNase activity (Korennykh and Walter, 2012). IRE1 α RNase activity cleaves a part of *XBPIu* mRNA (u; unspliced) to produce *XBPIs* (s; spliced) mRNA. After translation, the XBPIs protein enters the nucleus and acts as a transcription factor to induce the transcription of ER chaperones and the components of the ERAD machinery (Kimata and Kohno, 2011; Ron and Walter, 2007).

IRE1 α is ubiquitously expressed in various mammalian tissues. It is particularly highly expressed and activated in the pancreas (Iwawaki *et al.*, 2004; Tirasophon *et al.*, 1998; Tsuchiya Y., Saito M., Kadokura H., Miyazaki J., Tahiro F., Imagawa Y., Iwawaki T., Kohno K. submitted). However, the precise roles of IRE1 α and XBPIs/u in pancreatic β -cells remain undefined. Lipson *et al.* reported that the depletion of IRE1 α by siRNA and the inhibition of IRE1 α phosphorylation by the overexpression of its phosphorylation-deficient mutant (K599A) impaired proinsulin protein synthesis (Lipson *et al.*, 2006). They proposed that proinsulin biosynthesis is dependent on the phosphorylation of IRE1 α , rather than XBPIs production, as *XBPIs* mRNA expression was not increased by “physiological” levels of ER stress induced by high glucose (Lipson *et al.*, 2006). On the other hand, Hassler *et al.* reported that the overexpression of the K907A IRE1 α mutant that lacks RNase activity, decreases proinsulin protein synthesis (Hassler *et al.*, 2015), indicating that the RNase domain of IRE1 α also plays an important role in proinsulin biosynthesis. However, the precise role of the RNase domain of IRE1 α in insulin secretion remains unknown.

4 μ 8C (4-methyl umbelliferone 8-carbaldehyde) inhibits IRE1 α RNase activity (Cross *et al.*, 2012) by covalently modifying the K907 lysine residue in the RNase domain. It inhibits *XBPIu* mRNA splicing, but does not inhibit RNase L, which supports the idea that 4 μ 8C is a specific inhibitor of IRE1 α RNase activity (Cross *et al.*, 2012). Here, to examine whether IRE1 α RNase activity has a direct role in insulin biosynthesis and secretion, we employed 4 μ 8C and analyzed insulin secretory pathways and *XBPIu* mRNA splicing. We found that 4 μ 8C inhibited insulin exocytosis independent of IRE1 α RNase activity.

Materials and Methods

Cell culture

MIN6 (IRE1 $\alpha^{AR/AR}$) cells were established in our laboratory from pancreatic β -cells of IRE1 α conditional knockout mouse (Tsuchiya Y., Saito M., Kadokura H., Miyazaki J., Tahiro F., Imagawa Y., Iwawaki T., Kohno K. submitted). Briefly, IRE1 α RNase domain-floxed mice (Iwawaki *et al.*, 2009) were crossed with IT-6 mice, which expressed the SV 40 large T antigen under the control of an

insulin promoter (Miyazaki *et al.*, 1990, 2010). IRE1 α RNase domain-floxed mice whose pancreatic β -cells were immortalized by the large T antigen were obtained and pancreatic islets were isolated to establish MIN6 (IRE1 $\alpha^{fl/fl}$) cell lines. MIN6 (IRE1 $\alpha^{AR/AR}$) cells were IRE1 α RNase domain-deficient cells derived from MIN6 (IRE1 $\alpha^{fl/fl}$) cells transfected with Cre by adenovirus for 4 days. GFP-transfected MIN6 (IRE1 $\alpha^{fl/fl}$) cells were used as controls. MIN6 (IRE1 $\alpha^{fl/fl}$) and MIN6 (IRE1 $\alpha^{AR/AR}$) cells were maintained in culture medium (DMEM, 4.5 g/l glucose (Nacalai, Kyoto, Japan) supplemented with 15% FBS (Corning, lot. 35010117), 55 μ M 2-mercaptoethanol, 100 U/ml penicillin, and 100 μ g/ml streptomycin) at 37°C in a 5% CO₂ incubator.

Animals

All experimental protocols involving animals were approved by the Committee on Animal Research at Nara Institute of Science and Technology (NAIST) and were carried out in accordance with the institutional guidelines of NAIST.

Plasmids and primers

To generate the plasmids pcDNA3.1(+) mXBPI unspliced form and pcDNA3.1(+) mXBPI spliced form, the unspliced form of mouse *XBPI* (accession number: NM013842) and the spliced form of mouse *XBPI* (accession number: AF443192) cDNAs were constructed by RT-PCR using total RNA prepared from the mouse liver treated with tunicamycin for 6 hr. They were digested with HindIII and XbaI and ligated with the pcDNA3.1(+) vector digested with HindIII and XbaI. Two primers were used to determine *XBPIu* mRNA splicing by PCR, 5'-GAGAACCAGGAGTT-AAGAACACG-3' and 5'-GAAGATGTTCTGGGGAGGTGAC-3'.

XBPIu mRNA splicing

A total of 4.0×10^6 MIN6 (IRE1 $\alpha^{fl/fl}$) cells were plated in a 6-well plate and incubated in culture medium for 4 days. The cells were treated with DMSO or 4 μ 8C (TOCRIS, Ellisville, MO, USA) at the indicated concentration for 5 hr. After 1 hr, they were treated with 1 mM DTT and either DMSO or 4 μ 8C. RNA was extracted from these cells using RNAiso Plus (TaKaRa, Shiga, Japan) and RT-PCR was performed with mouse *XBPIu* primers. *XBPIu* and *XBPIs* plasmids were used as controls. The PCR products were run on a 5% acrylamide gel and stained with ethidium bromide. Images were obtained using ImageQuant LAS 4000 (GE Healthcare, Chicago, IL, USA). *XBPIu* mRNA splicing was examined in GFP-transfected MIN6 (IRE1 $\alpha^{fl/fl}$) and MIN6 (IRE1 $\alpha^{AR/AR}$) cells following the same methods.

To analyze the time-course of *XBPIu* mRNA splicing after high glucose stimulation, 4.0×10^6 MIN6 (IRE1 $\alpha^{fl/fl}$) cells were plated in a 6-well plate and incubated in culture medium for 4 days. The cells were washed 3 times with KRBH buffer (115 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl₂·6H₂O, 1.2 mM NaH₂PO₄, 2.5 mM CaCl₂, 25 mM NaHCO₃, 10 mM HEPES, and 2 mg/ml BSA) and incubated in low glucose medium (1.67 mM glucose/KRBH) for 2

hr. The medium was changed to low or high glucose medium (16.7 mM glucose/KRBH) with DMSO or 64 μ M 4 μ 8C and the cells were incubated for indicated times. *XBP1u* mRNA splicing was examined as described above. The PCR products were run on a 7.5% acrylamide gel and stained with ethidium bromide; images were obtained using Gel Doc XR+ (Bio-Rad, Hercules, CA, USA).

ELISA

8.0 $\times$ 10⁶ MIN6 (*Ire1 α ^{fl/fl}*) cells were plated in a 6-cm dish and incubated in culture medium for 4 days. The cells were washed 3 times with KRBH buffer and incubated in low glucose medium with DMSO or 4 μ 8C for 2 hr. The medium was changed to low or high glucose medium with DMSO or 4 μ 8C and the cells were incubated for indicated times. The insulin concentration in the medium was determined using the Mouse Insulin ELISA KIT (AKRIN-011T; Shibayagi, Gunma, Japan). Total insulin in the medium was determined by multiplying the insulin concentration by the total volume of the medium. The cells were washed twice with PBS, lysed with lysis buffer (2% SDS, 100 mM Tris-HCl, pH 6.8), and centrifuged at the maximum speed of a microcentrifuge for 10 min. The cell lysate was prepared from the supernatant and its protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The total protein amount was determined by multiplying the protein concentration of the cell lysate by the total volume of the cell lysate. Insulin secretion was calculated by dividing the total insulin amount in the medium by the total protein amount in the cell lysate. To analyze the effect of 4 μ 8C on insulin secretion in a short time period, the experiments were performed with following modifications. A total of 4.0 $\times$ 10⁶ MIN6 (*Ire1 α ^{fl/fl}*) cells were plated in a 6-well plate and incubated in culture medium for 4 days. The cells were washed 3 times with KRBH buffer and incubated in low glucose medium for 2 hr without 4 μ 8C. The cells were incubated for indicated times with or without 4 μ 8C and insulin secretion was quantitatively evaluated by ELISA.

SEAP secretion

A total of 4.8 $\times$ 10⁶ pancreatic β -cells were transfected with a plasmid carrying secretory alkaline phosphatase (SEAP, kindly provided by Dr. Paul Randazzo, National Cancer Institute, Bethesda, MD, USA) with Lipofectamine 2000 by reverse transfection, following the manufacturer's protocol (Thermo Fisher Scientific). They were plated on a 6-well plate. Two days after transfection, the cells were washed twice and incubated with 1 ml of culture medium for times indicated in Fig. 1F at 37°C with either DMSO or 4 μ 8C. Then, 50 μ l of culture medium was collected to determine the amount of secreted SEAP. The cells were washed twice with PBS and lysed in 200 μ l of lysis buffer (0.1% Triton X-100, 1 $\times$ dilution buffer). Then, 10 μ l of lysate was obtained to determine the amount of SEAP in the cell lysate. The medium and the lysate were processed as per the manufacturer's protocol (Phospha-LightTM SEAP Reporter Gene Assay System; Thermo

Fisher Scientific). Chemiluminescence was measured using the TriStar2 LB 942 (Berthold, Bad Wilbad, Germany). SEAP secretion was calculated by dividing the amount of SEAP in the medium by the total amount of SEAP in the medium and the lysate.

Pulse experiments

A total of 4.0 $\times$ 10⁶ MIN6 (*Ire1 α ^{fl/fl}*) cells were plated in a 6-well plate and incubated in culture medium for 4 days. To examine the effect of 4 μ 8C under low glucose treatment, cells were washed 3 times with KRBH buffer and incubated in low glucose medium with or without 4 μ 8C for 1 hr. The medium was changed to low glucose medium including 110 μ Ci/ml [³⁵S]-Cys/Met (Perkin-Elmer, NEG772) with or without 4 μ 8C and incubated for 30 min. To examine the effect of 4 μ 8C under high glucose treatment, the cells were washed 3 times with KRBH buffer and incubated in low glucose medium with or without 4 μ 8C for 2 hr. The medium was changed to high glucose medium including 110 μ Ci/ml [³⁵S]-Cys/Met with or without 4 μ 8C and incubated for 30 min. The cells were washed twice with PBS, lysed with lysis buffer (2% SDS, 100 mM Tris-HCl, pH 6.8), and centrifuged at the maximum speed of the microcentrifuge for 10 min. The cell lysate was prepared from the supernatant and its protein concentration was determined using the Pierce BCA Protein Assay Kit. Then, 4 $\times$ sample buffer was added to the cell lysate, followed by treatment with 10% 2-mercaptoethanol. The samples were boiled at 98°C for 10 min and run on a NuPAGE 4–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA, USA). Labeled proteins were detected using the BAS-2500 (FujiFilm, Tokyo, Japan).

Pulse-chase experiments

The pulse experiments were performed using the methods described above with the following modifications. Cells were pretreated with low glucose medium for 2 hr with or without 4 μ 8C. The medium was changed to high glucose medium containing 110 μ Ci/ml [³⁵S]-Cys/Met with or without 4 μ 8C and the cells were labeled for 15 min. After pulse labeling, the medium was changed to high glucose medium with or without 4 μ 8C and the cells were chased for indicated times.

Islets

Pancreatic islets from 8-week-old C57BL/6J male mice were plated in a 6-well transwell plate with 40–50 islets/well and incubated overnight in the culture medium (DMEM, 4.5 g/l glucose (Nacalai) supplemented with 15% FBS, 55 μ M 2-mercaptoethanol, 100 U/ml penicillin, and 100 μ g/ml streptomycin). Transwells were used to wash islets to minimize physical stimulation. When islets were washed, the medium was added to both the transwell and the plate, and the medium was aspirated from the plate. The islets were washed 3 times with KRBH buffer and incubated in low glucose medium for 2 hr. The medium was changed to low or high glucose medium and the islets were incu-

Fig. 1. 4μ8C inhibits insulin secretion within 2 hr after high glucose treatment. **A.** MIN6 (*Ire1α^{fl/fl}*) cells were treated with the indicated concentration of 4μ8C for 6 hr and 1 mM DTT for 1 hr with 4μ8C. *XBP1u* mRNA splicing was determined by RT-PCR with *XBP1* primers. **B.** Cells were stimulated by high glucose (16.7 mM) and treated with 64 μM 4μ8C for the indicated times and analyzed as in **A**. The panel shows representative results of three independent experiments. **C.** The graph shows the ratio between *XBP1s* and total *XBP1(s+u)*. Data are presented as averages of three independent experiments with S.D. **p* < 0.05, ***p* < 0.01, n.s.; not significant. **D.** Cells were incubated with low (1.67 mM) or high glucose (16.7 mM) medium for the indicated times with or without 64 μM 4μ8C. The secreted insulin level in the medium was measured by ELISA and normalized by the total protein amount. Data are presented as averages of three independent experiments with S.D. **E.** The graph shows a comparison of secreted insulin levels between control and 4μ8C-treated cells under high glucose condition. Student's *t*-test was used to analyze statistical significance. **p* < 0.05, n.s.; not significant. **F.** SEAP secretion was determined by measuring secreted SEAP in the medium and normalized by total SEAP in the medium and lysate. The mean of three experiments is shown. n.s.; not significant.

bated with DMSO or 4 μ 8C. The islets were incubated for indicated times and insulin secretion was quantitatively evaluated by ELISA. In these experiments, the medium was changed every time samples were obtained for ELISA.

Results

4 μ 8C inhibits insulin secretion within 2 hr

To examine the effects of 4 μ 8C in our pancreatic β -cells (i.e., MIN6 (*Ire1a*^{fl/fl}) cells), we first determined the 4 μ 8C concentration that inhibits *XBPLu* mRNA splicing in these cells. We treated pancreatic β -cells with 4 μ 8C at concentrations of 16, 32, and 64 μ M for 6 hr and examined if *XBPLu* mRNA splicing induced by 1 mM DTT was inhibited. As shown in Fig. 1A, 64 μ M 4 μ 8C inhibited 87.1% of *XBPLu* mRNA splicing without DTT and 72.1% with DTT. Next, we analyzed the time-course of *XBPLu* mRNA splicing after high (16.7 mM) glucose stimulation with or without 4 μ 8C. As shown in Fig. 1B, high glucose stimulation increased *XBPLu* mRNA splicing 2.4-fold at 15 and 30 min compared with that at 0 min. By treatment of 64 μ M 4 μ 8C, *XBPLu* mRNA splicing was inhibited by 69.5% at 15 min and 79.7% at 30 min compared with that observed in DMSO-treated cells (Fig. 1B and 1C). These results indicate that 64 μ M 4 μ 8C inhibits *XBPLu* mRNA splicing by more than ~70% in our cells.

Next, we analyzed the effects of 4 μ 8C on insulin secretion. We treated pancreatic β -cells with either DMSO (control) or 64 μ M 4 μ 8C in low (1.67 mM) or high (16.7 mM) glucose medium and examined insulin secretion. In control cells, insulin secretion was stimulated in high glucose medium, but not in low glucose medium (Fig. 1D, left panel). Surprisingly, 64 μ M 4 μ 8C inhibited 95.8% of insulin secretion under high glucose within 2 hr (Fig. 1D, right panel and Fig. 1E). We also analyzed constitutive secretion by SEAP (secretory alkaline phosphatase) secretion. We transfected pancreatic β -cells with SEAP cDNA and examined SEAP secretion with or without 4 μ 8C (Fig. 1F). SEAP secretion was not perturbed by 4 μ 8C. Our results indicate that 4 μ 8C specifically impaired insulin secretion, but not constitutive secretion.

4 μ 8C does not inhibit insulin biosynthesis and maturation

To determine the process by which 4 μ 8C inhibits insulin secretion, we first investigated insulin protein synthesis after treatment with 4 μ 8C. As shown in Fig. 2A, pancreatic β -cells were labeled with [³⁵S]-Cys/Met and treated with 4 μ 8C for 30 min, and cell lysates were prepared for autoradiography. Although the amount of synthesized insulin increased by high glucose stimulation, the amount of total synthesized protein also increased (Fig. 2A). We quantified

proinsulin, insulin, and total synthesized protein, as indicated in Fig. 2B, and calculated insulin synthesis as described in the Materials and Methods (Fig. 2C). There was no significant difference in insulin synthesis between cells treated with or without 4 μ 8C. Second, we investigated insulin maturation after treatment with 4 μ 8C. Pancreatic β -cells were labeled with [³⁵S]-Cys/Met for 15 min in high glucose and chased for 30, 60, and 120 min with or without 4 μ 8C. The medium and the cell lysates were prepared for autoradiography (Fig. 2D). We quantified proinsulin and insulin amounts in the medium and the cell lysates, as shown in Fig. 2E, and determined insulin maturation as described in Fig. 2F and the Materials and Methods. Our results showed that insulin maturation was not significantly affected by 4 μ 8C (Fig. 2F). We concluded that 4 μ 8C does not inhibit insulin protein synthesis and maturation in our conditions.

4 μ 8C inhibited insulin secretion within 5 min

4 μ 8C inhibited ~90% of insulin secretion within 2 hr of high glucose treatment (Fig. 1D and 1E). However, there was no significant perturbation in insulin biosynthetic pathways (Fig. 2). Therefore, we examined the possibility that 4 μ 8C blocked insulin exocytosis, the final step of insulin secretion. Insulin exocytosis occurs within 5 min of exposure to a stimulus (Sugawara *et al.*, 2009; Xie *et al.*, 2015; Zhu *et al.*, 2013). We treated pancreatic β -cells with high glucose for 5, 10, 15, and 30 min and examined insulin secretion under 4 μ 8C (Fig. 3A, 3B, and 3C). In control conditions, high glucose stimulated insulin secretion within 5 min (Fig. 3A), even though the level of secreted insulin was low. In 4 μ 8C-treated cells, 85.4% of insulin secretion was inhibited in 5 min (Fig. 3B and 3C). To confirm that 4 μ 8C impairs insulin secretion within 5 min, we examined pancreatic islets from mice. Although insulin secretion from islets in control conditions exhibited variation, we repeatedly detected inhibitory effects of 4 μ 8C on insulin secretion within 5 min after high glucose treatment (Fig. 3D, Ex. 1, 2, and 3). Student's *t*-tests (Fig. 3E) and two-way ANOVA (data not shown) both showed statistically significant differences between 4 μ 8C-treated and control cells at 5 min. These results indicate that 4 μ 8C inhibits insulin secretion in 5 min.

4 μ 8C inhibits insulin secretion independent of the RNase domain of IRE1 α

We found that 4 μ 8C inhibited insulin secretion at 5 min after high glucose treatment in our cells and in mouse islets. At this time point, pre-formed insulin granules can fuse with the plasma membrane upon high glucose stimulation, whereas newly synthesized proinsulin requires a longer time period for secretion (Sugawara *et al.*, 2009; Wang *et al.*, 2011; Xie *et al.*, 2015; Zhu *et al.*, 2013). Our data imply that 4 μ 8C inhibits insulin exocytosis. However, at 5

Fig. 2. 4μ8C does not affect insulin biosynthesis and maturation. A. Cells were incubated with low or high glucose medium containing 110 μCi/ml with or without 4μ8C and incubated for 30 min. Radiolabeled proteins from whole cell lysates are shown. The panel shows representative results of three independent experiments. B. The results shown in A were quantified to determine total protein, proinsulin, or insulin, as indicated. C. The graph shows synthesized insulin normalized by synthesized total protein. Insulin synthesis in low glucose was set as 1. The graph shows the average of three independent experiments with S.D. n.s.; not significant. D. Cells were incubated with high glucose medium including 110 μCi/ml [³⁵S]-Cys/Met with or without 4μ8C for 15 min. After labeling, the medium was changed to high glucose medium with or without 4μ8C and the cells were chased for the indicated times. Radiolabeled proteins from the medium and the whole cell lysates are shown. The panel shows representative results of three independent experiments. E. The results shown in D were quantified as indicated to determine proinsulin and insulin. F. The graph shows a comparison of insulin maturation between control and 4μ8C-treated cells. The equation to calculate insulin maturation is indicated above. The graph shows the average of three independent experiments with S.D. n.s.; not significant.

min of treatment with 4μ8C, there was no apparent inhibition of *XBPlu* mRNA splicing (Fig. 1B). This result implies that 4μ8C inhibits insulin exocytosis independent of *XBPlu* mRNA splicing. We examined whether 4μ8C inhibits insulin secretion independent of the RNase domain of IRE1α using pancreatic β-cells that lack the RNase domain of IRE1α (MIN6 (*Ire1α^{AR/AR}*) cells), which were established by our group (see Materials and Methods). Control GFP-transfected MIN6 (*Ire1α^{fl/fl}*) cells showed insulin secretion in high glucose medium, similar to non-transfected cells (Fig. 4A and 4B). In contrast, Cre-

transfected MIN6 (*Ire1α^{AR/AR}*) cells showed more than 50% inhibition of insulin secretion at all time points after high glucose stimulation compared with secretion by GFP-transfected MIN6 (*Ire1α^{fl/fl}*) cells (Fig. 4C). Our results support the idea that IRE1α RNase activity is required for insulin secretion (Hassler *et al.*, 2015). Next, we treated MIN6 (*Ire1α^{AR/AR}*) cells with 4μ8C (Fig. 4D). We observed strong inhibition of insulin secretion, even in MIN6 (*Ire1α^{AR/AR}*) cells (more than 74.8% inhibition) (Fig. 4C, 4D, and 4E). These results indicate that 4μ8C blocks insulin secretion, beyond the effect of the depletion of the RNase

Fig. 3. 4 μ 8C inhibits insulin secretion within 5 min after high glucose treatment. **A.** MIN6 (*Ire1 α ^{fl/fl}*) cells were incubated with low or high glucose medium for indicated times. The insulin secretion was measured by ELISA as in Fig. 1D. Data are presented as the average of four independent experiments with S.D. **B.** MIN6 (*Ire1 α ^{fl/fl}*) cells were incubated with low or high glucose medium for the indicated times with 4 μ 8C. Data are presented as the average of four independent experiments with S.D. **C.** The graph shows a comparison of insulin secretion in control and 4 μ 8C-treated cells under high glucose condition. Student's *t*-test was used to analyze statistical significance. **p* < 0.05, n.s.; not significant. **D.** Mice pancreatic islets were incubated with low or high glucose medium for indicated times with 4 μ 8C. The insulin secretion was measured as in A. Ex.1, 2, and 3 show the results of three independent experiments. **E.** The graph shows a comparison of secreted insulin levels under high glucose conditions between control and 4 μ 8C-treated islets. The graph shows the average of six independent experiments with S.D. Student's *t*-test was used to analyze the statistical significance between control and 4 μ 8C treatment. **p* < 0.05, ***p* < 0.01, n.s.; not significant.

Fig. 4. 4μ8C inhibits insulin secretion in cells deficient in the RNase domain of IRE1α. **A.** MIN6 (*Ire1α^{fl/fl}*) cells were incubated with low or high glucose medium for the indicated times. The insulin secretion was measured by ELISA as in Fig. 1D. Data are presented as the average of three independent experiments with S.D. **B.** The graph shows insulin secretion in MIN6 (*Ire1α^{fl/fl}*) cells transfected with GFP by adenovirus as a control. The cells were treated as in **A**. **C.** MIN6 (*Ire1α^{fl/fl}*) cells were transfected with Cre by adenovirus to make the cells deficient in the RNase domain of IRE1α (MIN6 (*Ire1α^{ΔR/ΔR}*)). The graph shows insulin secretion in MIN6 (*Ire1α^{ΔR/ΔR}*) cells. The cells were treated as in **A**. **D.** MIN6 (*Ire1α^{ΔR/ΔR}*) cells were incubated with low or high glucose medium for the indicated times with 4μ8C. The insulin secretion was measured as in **A**. Data are presented as the average of three independent experiments with S.D. **E.** The graph shows a comparison of secreted insulin levels in 4μ8C-treated or DMSO-treated MIN6 (*Ire1α^{ΔR/ΔR}*) cells under high glucose conditions. The graph shows the average of three independent experiments with S.D. Student's *t*-test was used to analyze the statistical significance. **p* < 0.05, ***p* < 0.01, n.s.; not significant. **F.** Non-transfected and GFP-transfected MIN6 (*Ire1α^{fl/fl}*) cells and MIN6 (*Ire1α^{ΔR/ΔR}*) cells were treated with 4μ8C for 6 hr and 1 mM DTT for 1 hr with 4μ8C. *XBP1u* mRNA splicing was determined by RT-PCR with *XBP1* primers. The panel shows *XBP1u* and *XBP1s* mRNAs. Note that MIN6 (*Ire1α^{ΔR/ΔR}*) cells did not show any residual *XBP1s* mRNA without 4μ8C.

domain of IRE1 α . To confirm that there is no residual *XBPLu* mRNA splicing activity in MIN6 (*Ire1 α ^{AR/AR}*) cells, we treated MIN6 (*Ire1 α ^{AR/AR}*) cells with 1 mM DTT and examined *XBPLu* mRNA splicing (Fig. 4F). MIN6 (*Ire1 α ^{AR/AR}*) cells did not show any *XBPLu* mRNA splicing, even under DTT treatment, supporting the idea that MIN6 (*Ire1 α ^{AR/AR}*) cells do not have any residual *XBPLu* mRNA splicing activity. 4 μ 8C did not show further inhibition of *XBPLu* mRNA splicing in MIN6 (*Ire1 α ^{AR/AR}*) cells (Fig. 4F). These results indicate that 4 μ 8C inhibits insulin secretion independent of RNase activity of IRE1 α .

Discussion

In this study, we found a strong inhibitory effect of 4 μ 8C on insulin secretion (Fig. 1D and 1E), independent of insulin protein synthesis and maturation (Fig. 2). We further found that 4 μ 8C inhibited insulin secretion within 5 min of high glucose stimulation (Fig. 3), suggesting that 4 μ 8C inhibits insulin exocytosis. However, IRE1 α is localized in the ER (Tirasophon *et al.*, 1998), and 4 μ 8C took at least 15 min to inhibit *XBPLu* mRNA splicing induced by high glucose stimulation (Fig. 1B and 1C). Using MIN6 (*Ire1 α ^{AR/AR}*) cells, we further found that the inhibition of insulin secretion by 4 μ 8C is not dependent on the RNase domain of IRE1 α (Fig. 4C, 4D, and 4E). Our work revealed that 4 μ 8C has off-target effects, and might inhibit other molecule(s) in addition to the RNase domain of IRE1 α in pancreatic β -cells to inhibit insulin exocytosis.

4 μ 8C is a widely-used inhibitor of IRE1 α (Cross *et al.*, 2012; Eletto *et al.*, 2016; Harding *et al.*, 2012; Sharma *et al.*, 2015). However, its off-target effects have not been reported. Our work indicates that the existence of other targets of 4 μ 8C should be considered when using “crude” experimental systems, such as living cells or cell lysates. We tested the lower concentration of 4 μ 8C for *XBPLu* mRNA splicing and insulin secretion. We found that 32 μ M of 4 μ 8C inhibited 43% of *XBPLu* mRNA splicing and 16 μ M inhibited 26% compared with that in DMSO-treated cells in the presence of DTT (Fig. 1A). Whereas we observed 32 μ M 4 μ 8C still inhibited 84% of insulin secretion, and 16 μ M inhibited 71% (data not shown). That is, even if we used 16 μ M of 4 μ 8C that largely did not inhibit *XBPLu* mRNA splicing, 71% of insulin secretion was impaired. These results imply that the off-target effects are stronger than the effect of inhibition of RNase activity of IRE1 α . We also tested STF-083010, another inhibitor of IRE1 α RNase activity (Papandreou *et al.*, 2011). STF-083010 also blocked insulin secretion within 5 min after high glucose stimulation (data not shown). STF-083010 could inhibit both the RNase activity of IRE1 α and other target(s) in pancreatic β -cells, similar to 4 μ 8C, because these reagents have similar structures.

We did not detect any inhibitory effects of 4 μ 8C on insu-

lin biosynthesis and the maturation process in our conditions (Fig. 2), whereas the overexpression of the K907A mutant of IRE1 α has been reported to inhibit insulin biosynthesis (Hassler *et al.*, 2015). In the latter case, the K907A mutant was overexpressed for 72 hr. This condition might be sufficient to change the gene expression profiles downstream of XBP1s expression, possibly leading to the inhibition of insulin biosynthesis. In contrast, we tested insulin biosynthesis and maturation within 2 hr of high glucose treatment with 4 μ 8C. We pretreated cells with 4 μ 8C in low glucose medium for 1 or 2 hr before high glucose treatment; the maximum treatment times with 4 μ 8C were 2 hr 30 min in the pulse experiment (Fig. 2A–2C) and 4 hr 15 min in the pulse-chase experiment (Fig. 2D and 2E). These durations are much shorter than 72 hr (in the K907A overexpression experiment). We speculate that 4 μ 8C enabled us to determine the effects that were independent of the changes in gene expression. The knockdown, knockout or overexpression method usually requires more than 12 hr to see its effects, therefore it is often difficult to distinguish the short-term effects (such as the structural changes of RNase domain) from the long-term effects (such as the change of gene expression profiles). Our experiments showed that 15 min is sufficient to inhibit IRE1 α RNase activity with 4 μ 8C (Fig. 1B and 1C). Long-term treatment (e.g., 72 hr) with 4 μ 8C might inhibit insulin biosynthesis or maturation, similar to K907A overexpression.

The inhibitors are useful tools owing to their ability to inhibit IRE1 α RNase activity in a short time period, without requiring genetic manipulations. They enable us to analyze the direct function of the IRE1 α RNase domain, without considering changes in gene transcription and translation. The identification of more specific inhibitors of the IRE1 α RNase domain that lack off-target effects is necessary for more precise analyses of IRE1 α function.

Acknowledgments. We thank the members of the Kohno lab for helpful suggestions and discussion as well as Hisayo Masuda, Masami Yoshida, and Azumi Wada for technical assistance. This work was supported by the Japan Society for the Promotion of Science (JSPS) [Grant-in-Aid for Young Scientists, Start-up, JP15H06410 to SY, Grant-in-Aid for Scientific Research (C), JP26430090 to MS, Grant-in-Aid for Scientific Research (S), JP24228002 and Grant-in-Aid for Scientific Research on Innovative Areas, JP26116006 to KK].

References

- Cross, B.C., Bond, P.J., Sadowski, P.G., Jha, B.K., Zak, J., Goodman, J.M., Silverman, R.H., Neubert, T.A., Baxendale, I.R., Ron, D., *et al.* 2012. The molecular basis for selective inhibition of unconventional mRNA splicing by an IRE1-binding small molecule. *Proc. Natl. Acad. Sci. USA*, **109**: E869–E878.
- Eletto, D., Eletto, D., Boyle, S., and Argon, Y. 2016. PDIA6 regulates insulin secretion by selectively inhibiting the RIDD activity of IRE1. *FASEB J.*, **30**: 653–665.
- Harding, H.P., Zyryanova, A.F., and Ron, D. 2012. Uncoupling proteostasis and development in vitro with a small molecule inhibitor of the pancreatic endoplasmic reticulum kinase, PERK. *J. Biol. Chem.*, **287**:

- 44338–44344.
- Hassler, J.R., Scheuner, D.L., Wang, S., Han, J., Kodali, V.K., Li, P., Nguyen, J., George, J.S., Davis, C., Wu, S.P., *et al.* 2015. The IRE1alpha/XBP1s Pathway Is Essential for the Glucose Response and Protection of beta Cells. *PLoS Biol.*, **13**: e1002277.
- Iwawaki, T., Akai, R., Kohno, K., and Miura, M. 2004. A transgenic mouse model for monitoring endoplasmic reticulum stress. *Nat. Med.*, **10**: 98–102.
- Iwawaki, T., Akai, R., Yamanaka, S., and Kohno, K. 2009. Function of IRE1 alpha in the placenta is essential for placental development and embryonic viability. *Proc. Natl. Acad. Sci. USA*, **106**: 16657–16662.
- Kimata, Y. and Kohno, K. 2011. Endoplasmic reticulum stress-sensing mechanisms in yeast and mammalian cells. *Curr. Opin. Cell Biol.*, **23**: 135–142.
- Korennykh, A. and Walter, P. 2012. Structural basis of the unfolded protein response. *Annu. Rev. Cell Dev. Biol.*, **28**: 251–277.
- Lipson, K.L., Fonseca, S.G., Ishigaki, S., Nguyen, L.X., Foss, E., Bortell, R., Rossini, A.A., and Urano, F. 2006. Regulation of insulin biosynthesis in pancreatic beta cells by an endoplasmic reticulum-resident protein kinase IRE1. *Cell Metab.*, **4**: 245–254.
- Liu, M., Wright, J., Guo, H., Xiong, Y., and Arvan, P. 2014. Proinsulin entry and transit through the endoplasmic reticulum in pancreatic beta cells. *Vitam. Horm.*, **95**: 35–62.
- Miyazaki, J., Araki, K., Yamato, E., Ikegami, H., Asano, T., Shibasaki, Y., Oka, Y., and Yamamura, K. 1990. Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. *Endocrinology*, **127**: 126–132.
- Miyazaki, S., Taniguchi, H., Moritoh, Y., Tashiro, F., Yamamoto, T., Yamato, E., Ikegami, H., Ozato, K., and Miyazaki, J. 2010. Nuclear hormone retinoid X receptor (RXR) negatively regulates the glucose-stimulated insulin secretion of pancreatic ss-cells. *Diabetes*, **59**: 2854–2861.
- Papandreou, I., Denko, N.C., Olson, M., Van Melckebeke, H., Lust, S., Tam, A., Solow-Cordero, D.E., Bouley, D.M., Offner, F., Niwa, M., *et al.* 2011. Identification of an IRE1alpha endonuclease specific inhibitor with cytotoxic activity against human multiple myeloma. *Blood*, **117**: 1311–1314.
- Rhodes, C.J., Shoelson, S., and Halban, P.A. 2005. Insulin Biosynthesis, Processing, and Chemistry. In: Joslin's Diabetes Mellitus (C.R. Kahn, G.C. Weir, G.L. King, A.M. Jacobson, A.C. Moses, and R.J. Smith, eds.). Boston: Joslin Diabetes Center, Chapter 5: 65–82.
- Ron, D. and Walter, P. 2007. Signal integration in the endoplasmic reticulum unfolded protein response. *Nat. Rev. Mol. Cell Biol.*, **8**: 519–529.
- Schuit, F.C., In't Veld, P.A., and Pipeleers, D.G. 1988. Glucose stimulates proinsulin biosynthesis by a dose-dependent recruitment of pancreatic beta cells. *Proc. Natl. Acad. Sci. USA*, **85**: 3865–3869.
- Sharma, R.B., O'Donnell, A.C., Stamateris, R.E., Ha, B., McCloskey, K.M., Reynolds, P.R., Arvan, P., and Alonso, L.C. 2015. Insulin demand regulates beta cell number via the unfolded protein response. *J. Clin. Invest.*, **125**: 3831–3846.
- Sugawara, K., Shibasaki, T., Mizoguchi, A., Saito, T., and Seino, S. 2009. Rab11 and its effector Rip11 participate in regulation of insulin granule exocytosis. *Genes Cells*, **14**: 445–456.
- Sun, J., Cui, J., He, Q., Chen, Z., Arvan, P., and Liu, M. 2015. Proinsulin misfolding and endoplasmic reticulum stress during the development and progression of diabetes. *Mol. Aspects Med.*, **42**: 105–118.
- Tirasophon, W., Welihinda, A.A., and Kaufman, R.J. 1998. A stress response pathway from the endoplasmic reticulum to the nucleus requires a novel bifunctional protein kinase/endoribonuclease (Ire1p) in mammalian cells. *Genes Dev.*, **12**: 1812–1824.
- Van Lommel, L., Janssens, K., Quintens, R., Tsukamoto, K., Vander Mierde, D., Lemaire, K., Denef, C., Jonas, J.C., Martens, G., Pipeleers, D., *et al.* 2006. Probe-independent and direct quantification of insulin mRNA and growth hormone mRNA in enriched cell preparations. *Diabetes*, **55**: 3214–3220.
- Wang, H., Ishizaki, R., Kobayashi, E., Fujiwara, T., Akagawa, K., and Izumi, T. 2011. Loss of granuphilin and loss of syntaxin-1A cause differential effects on insulin granule docking and fusion. *J. Biol. Chem.*, **286**: 32244–32250.
- Xie, L., Zhu, D., Dolai, S., Liang, T., Qin, T., Kang, Y., Xie, H., Huang, Y.C., and Gaisano, H.Y. 2015. Syntaxin-4 mediates exocytosis of pre-docked and newcomer insulin granules underlying biphasic glucose-stimulated insulin secretion in human pancreatic beta cells. *Diabetologia*, **58**: 1250–1259.
- Zhu, D., Koo, E., Kwan, E., Kang, Y., Park, S., Xie, H., Sugita, S., and Gaisano, H.Y. 2013. Syntaxin-3 regulates newcomer insulin granule exocytosis and compound fusion in pancreatic beta cells. *Diabetologia*, **56**: 359–369.

(Received for publication, February 8, 2017, accepted, March 14, 2017
and published online, March 18, 2017)